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Studies on the Degradation and the Synthesis of Thiamine Phosphates. (II)

Enzymatic Synthesis of Thiamine Diphsophates (Cocarboxylase)

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Cocarboxylase was discovered by Lohmann[1], and it has been shown to play an important rôle in the anaerobic as well as aerobic decarboxylation of pyruvate. Cocarboxylase was synthesized by the chemical[2] and enzymatical methods[3]. Enzymatically, yeast[4] and liver[5] contain the enzyme which acts to transfer in the presence of ATP the pyrophosephate bond of ATP to thiamine. Its detailed mechanism of transphorylation is not yet elucidated. On the other hand, Lipmann[6] and Stadtman[7] reported that the phosphate bond of acetyl-phosphate (acetyl-P) is transferred to adenylic acid resulting formation of ADP, and then ATP is formed. This report deals with the mechanism of phosphorylation of thiamine in the presence of acetyl-P, which has a energy-rich phosphate bond.

EXPERIMENTAL

Preparation of acetyl-P (8). In a flask 20 ml. of isopropenyl acetate, 2.5 ml. of 85 % phosphoric acid and 0.1 ml. of concentrated H_2SO_4 were mixed under stirring. To the mixture kept at 25°C for 35 min., water was added to make 50 ml. of total volume. After it was adjusted to pH 5.0 with lithium hydroxide, excess of isopropenyl acetate was removed with ether, then the solution was brought to pH 8.0 with lithium hydroxide. After lithium phosphate was removed 5 times of its volume of cold EtOH were added to the solution, so white precipitate of acetyl-P was obtained. It was fractionated by addition of EtoH between the 0.5-5.0 times of its volume of EtOH, and employed. Estimation of the amounts of phosphorus and acetic acid showed 90 % of its purity.

Preparation of thiamine monophosphate. TTP was prepared by Karrer's method[9], and hydrolyzed at 100°C for 20 min. with 1-*N* HCl. By addition of small volume of acetone a clear viscous precipitate was resulted, which was removed. In the filtrate white needle-shaped crystals occurred, by addition of excess volume of acetone, which was proved to be TMP of nearly 100 % of purity by partition paperchromatography.

Estimation of phosphorus and acetyl-P in the test mixtures. Allen's method[10] was empolyed to estimate the phosphorus. If *Lactobacillus delbrückii* was contained with phosphorus in the test mixture, it became turbid. In this case molybdene blue was extracted with 10 ml. and then 5 ml. of isobutanol successively. Seventy percent EtOH was added till total volume became 25 ml., and then phosphorus was estimated.

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As acetyl-P contained in the test mixture was hydrolyzed spontaneously to a certain extent, inorganic phosphorus and acetyl-P were estimated as follows. To the mixture of acetyl-P and inorganic phosphorus, 30 % $CaCl_2$ solution was added, and then 1/3 times of its volume of EtOH were added, and the mixture was centrifuged. The supernatant of the centrifuged fluid contained only acetyl-P, and not inorganic phosphorus. In a half portion of the supernatant fluid total phosphorus of acetyl-P was estimated by Allen's method as described above. To the another half portion of the fluid 28 % solution of hydroxylamine was added and the mixture was colored with HCl-$FeCl_3$ solution[11]. Thus the color density of a definite amount of acetyl-P was determined easily by using spectrophotometer.

Paperchromatography of thiamine derivatives. On the paperchromatography a mixture of *n*-propanol-water-1 M acetate buffer (pH 5.0) (70 : 20 : 10) was used as a solvent[12] and Toyo Roshi No. 50 (40×2 cm.) as filterpaper. It was developed for 18 hours in the refrigerator. The spots were recognized by spraying Dragendorff's reagent[13], or by spraying the solution of 1 % KOH and of 0.1 % potassium ferricyanide and examining under the ultra violet ray. Rf values of thiamine, TMP and TDP were 0.5, 0.25 and 0.1, respectively.

Preparation of enzyme suspension of *Lactobacillus delbrückii.* In 100 ml. of malt solution, *Lact. delb.* was cultivated for 48 hours at 37°C. It was centrifuged, dried with acetone and suspended in 1ml. of 0.5 M acetate buffer of pH 6.2 at once, and used as enzyme source.

Test mixture. It contained 0.5 ml. of *Lact. delb.* suspesnion, 16.6 mg. of acetyl-P, 3.3 mg. of V B_1 and 0.3 mg. of $MgCl_2 \cdot 6H_2O$ mixed with 1.0 ml. of acetate buffer of pH 6.2. This test mixture was placed at 37°C for 90 min., and after boiling for 2 min., the formation of cocarboxylase (CoC) was examined. The mixture, which was heated in boiling water for 2 min. just after mixing, and which was not placed at 37°C, was tested as a control.

Manometric estimation of CO_2 formed in the enzyme system. In the Warburg's manometer, development of CO_2 was determined as follows. Fresh baker's yeast was spread and dried by an electric fan at room temperature. One gram of this dried yeast was suspended in 50 ml. of 0.1 M Na_2HPO_4 solution, washed by stirring for 12 min. and the mixture was centrifuged. After washing twice in this way, it was washed finally with 50 ml. of distilated water. The washed yeast was suspended in 10 ml. of 0.1 M phosphate buffer of pH 6.2 with NaF solution as an inhibitor, and 2.0 ml. of this suspension were used as an apoenzyme. In one side arm, 0.5 ml. of 1 % Na-pyruvate and 0.05 % $MgCl_2 \cdot 6H_2O$ mixture were added, and into the other 0.5 ml. of cocarboxylase solution (or the test mixture) were poured. To prevent the development of CO_2 from glucose[14], NaF solution was mixed with the apoenzyme buffer solution previously, to make 0.005 M of its final concentration.

RESULTS

1. Change of inorganic phosphorus and acetyl-P. As shown in Table 1., the

Table. 1. Change of phosphate before and after the incubation in 0.1ml. of test mixture.

Incubation	Not incubated	Incubated at 37°C, 90 min.
Total inorganic P (acetyl-P-inorganic P)	258μg	228μg
Phosphorus of acetyl-P	110μg	55μg
True inorganic P	148μg	173μg
1N-HCl Δ7P+total inorge, P	260μg	250μg
Labile P (Δ7P)	2μg	22μg

total amount of inorganic phosphorus and acetyl-P decreased in the test mixture incubated at 37°C for 90 min. than in that which was not incubated, while the amount of acetyl-P was remarkably diminished (Table 1). From these results of phosphorus distribution, it might be demonstrated that organic phosphorus

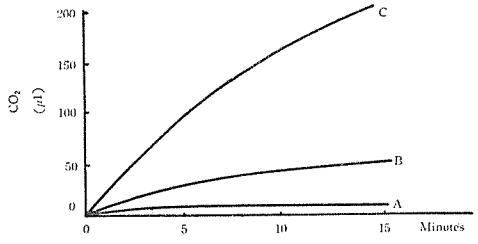

Fig. 1. Evolution of CO_2 from pyruvate,
A=Yeast apocarboxylase without V B_1,acetyl-P and *Lact. delb.*
B=V B_1+acetyl-P+heat-treated *Lact.delb.*+yeast apocarboxylase
C=V B_1+acetyl-P+*Lact. delb.*+yeast apocarboxylase

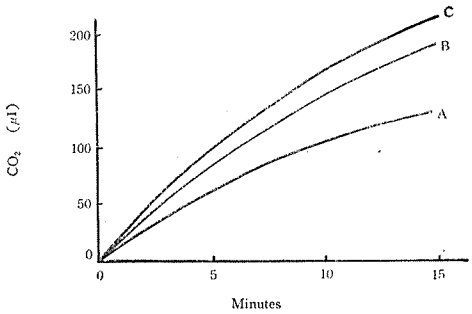

Fig. 2. Influences of $MgCl_2$ and NaF on the synthesis of CoC which was detected by the evolution of CO_2 from pyruvate.
A=NaF added in a final concentration of 0.05 M
B=Control without NaF
C=$MgCl_2$ added in a final cocentration of 0.001 M without NaF

compounds, especially 1 N-HCl Δ7P increased, which should suggest synthesis of cocarboxylase or ATP.

2. Paperchromatograph observation. A spot of V B_1 (Rf=0.5) and faint spots of TMP (Rf=0.25) and cocarboxylase (Rf=0.1) were recognized.

3. Manometric measurements of CO_2 decarboxylated from pyruvate by the cocarboxylase formed. As shown in Fig. 1, the evolution of CO_2 by the active enzyme system with cocarboxylase and yeast apoenzyme was greater than that by the heat-treated enzyme system. Mg^{++} activated the evolution of CO_2, while NaF inhibited it remarkably (Fig. 2).

4. Different effect of thiamine and thiamine monophosphate as a substrate. As shown in Fig. 3., V B_1 was more effective than TMP, but evolution of CO_2 was also recognized to a small extent in the incubation with TMP. It must be here noticed that when TMP was used as a substrate, faint spots of V B_1 and TDP were observed on partition paper chromatography.

5. Effect of organic phosphorus compounds in *Lact. delb.* Dried *Lact. delb.* incubated in 100 ml. of malt solution for 48 hours contained 500μg of inorganic phosphorus and 1800μg of organic phosphorus. In these organic phosphates, ATP, CoC and other energy-rich phosphate compounds might exist. In Fig. 4, results of experiments with V B_1 alone or with V B_1 and acetyl-P shown that when

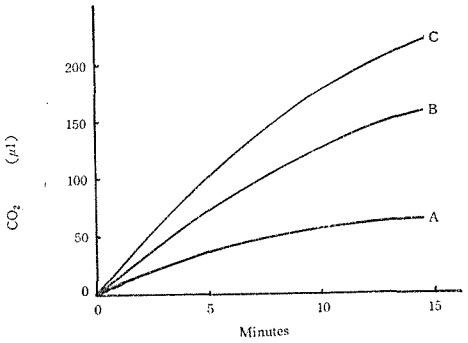

Fig. 3. Effect of thiamine and thiamine monophosphate as substrate on the synthesis of CoC which was detected by the evolution of CO_2 from pyruvate
A=heat-treated *Lact. delb.*
B=TMP+acetyl-P+*Lact. delb.*
C=V B_1+acetyl-P+*Lact. delb.*

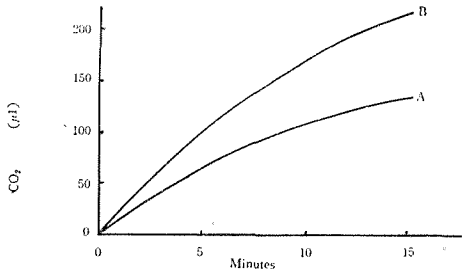

Fig. 4. Iufluence of acetyl-P on the sythesis of CoC which was detected by the evolution of CO_2 from pyruvate.
A=V B_1+*Lact. delb.* without acetyl-P,
B=V B_1+acety-P+*Lact. delb.*

acetyl-P was added, the evolution of CO_2 increased doubtly. Even if acetyl-P was not added, a little volume of CO_2 was developed. It seems to be due to some energy-rich phosphate compounds (as CoC, ATP) stored in the *Lact. delb.* prepration, which should be used in transphosphorylation or in some other enzyme systems, and this problem must be solved by further efforts.

DISCUSSION

Many animals and plants contain transphosphorylase. In the preliminary experiments, Takadiastase, potato apyrase and some sorts of bacteria (*Esch. coli* etc.) did not show the evolution of CO_2 in Warburg's aparatus (unpublished data). It was recognized, however, that acetyl-P was decomposed and Δ7p increased when Takadiastase and TMP were used, (unpublished date). It seems here that lgucose-1-P might be synthesized.

It is well known that in the animal tissues, acetyl-phosphatase was much stronger than *Lact. delb.* (about 10,000 times)[5], and existence of phosphotransacetylase was not yet ascertained. The synthesis of CoC in the presence of aectyl-P by animal tissue enzyme might be difficultly expected. In the present experiments the result indicates transphosphorylation to CoC was shown to occur in the presence of acetyl-P only by a specific bacterium such as *Lact. delb.* These experiments seemed to be very interesting to elucidate the mechanisms of transphosphorylation to CoC by *Lact. delb.* enzyme. It is questionable

whether TMP could act as a substrate in the sythesis of CoC by transphosphorylation, since when TMP was used as a substrate, the evolution of CO_2 was less than that when thiamine was used. It must be here remarked that the spot of thiamine was also recognized in PPC when CoC appeared to be synthesized in the presence of TMP.

So it must be supposed that TMP should be decomposed at first to thiamine, which was then readily synthesized to CoC. As already described *Lact. delb.* must contain some energy-rich phosphate compounds such as ATP, so it is assumable that ATP might play a rôle as phosphate donor in the CoC synthesis from thiamine. The details would be described in the proceeding paper.

SUMMARY

1. Cocarboxylase was synthesized from V B_1 in the presence of acetyl-P by the enzymes of *Lact. delb.* Evidence of CoC was confirmed by manometric observations in Warburg's apparatus, and by paperchromatographic method.

2. In the enzyme system with V B_1 and *Lact. delb.*, the evolution of CO_2 was also recognized without adding acetyl-P and it increased significantly if acetyl-P was added. The former phenomena might be due to some organic phosphate compounds of *Lact. delb.*

3. These results are consistent with the data that during the incubation the decomposition of acetyl-P and the increase of labile P were observed.

4. It is interesting that thiamine was more effective than thiamine monophosphate as a substrate in the formation of cocarboxylase by the enzyme system.

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